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13. ABSTRACT ***(Maximum 200 Words)***

The neuropeptide somatostatin is an important regulatory hormone that is widely distributed throughout the body. Somatostatin's actions are primarily inhibitory, and recently, it has been utilized as an antiproliferative agent against several tumor types, including breast neoplasms. Experimentally and clinically, somatostatin can inhibit breast cancer cell growth, possibly by inhibiting the secretion of growth factors, or by acting directly on the cells themselves to induce programmed cell death, or apoptosis. Despite increased clinical use, the mechanism(s) by which somatostatin acts to control breast cancer cell growth remain largely unknown. In this annual progress report, I describe studies performed on MDA231 cells, which show a robust response to somatostatin with regard to growth factor stimulation of the mitogen activated protein kinase, ERK1/2 (or MAPK). The activity of downstream components of the ras/raf/MAPK pathway, the Ets transcription factors, Ets-1 and Elk-1 are also inhibited by somatostatin. Furthermore, I show preliminary data indicating that another signaling pathway, the jun n-terminal kinase (JNK) pathway may be activated in response to somatostatin. . Together, these results begin to explain somatostatin's ability to inhibit cell growth and induce apoptosis in breast cancer. Furthermore, identification of these target pathways defines functional assays by which the efficacy of future anti-cancer drugs can be tested.

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FOREWORD

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John Tentler 1/3/00

PI Signature Date

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Introduction

The neuropeptide, somatostatin is an important regulatory hormone that is widely distributed throughout the body. For years it was recognized as the main negative regulator of growth hormone secretion from the anterior pituitary gland. Recently, however, it has become apparent that somatostatin can act at many tissues and organs, and in addition to its role as an inhibitor of hormone secretion, it is also capable of inhibiting cell growth and proliferation (1,2). As such, somatostatin is currently being investigated for its potential clinical use as an anti-cancer agent, as inhibition of cell growth is an important feature of most anti-cancer therapies. Indeed, stable somatostatin analogs, such as BIM23014 (Lanreotide) and SMS201-995 (Octreotide), are capable of inhibiting the growth of several types of tumors including pituitary adenomas, neuroblastomas, pancreatic cancer and breast cancer (2). At the cellular level, the actions of somatostatin are mediated by a family of G protein-coupled plasma membrane receptors termed ssts. To date, five distinct sst receptor subtypes have been identified, and these are named sst1-5 (3). Subtype 2 (sst2) is the most common somatostatin receptor found on tumor tissue, followed by sst5 (1, 4).

There is currently little information available regarding somatostatin action, especially as it pertains to breast neoplasms, and thus the objective of these studies is to define the functional role of somatostatin in several breast cancer cell lines as it impinges on growth factor-stimulated signaling pathways. Growth factors, such as EGF, FGF, and IGF-1, have been implicated in breast cancer tumorigenesis and progression (5), and therefore I plan to determine if these pathways are affected by somatostatin in a somatostatin-responsive breast cancer cell line. My underlying hypothesis is that somatostatin exerts its antiproliferative effects on a wide variety of tumors by interfering with multiple signaling pathways which lead to cell division.

Thus, my initial goals were to: 1) develop a somatostatin-responsive breast cancer cell model system, 2) to determine whether somatostatin inhibits growth factor- or UV light-stimulated Raf kinase, MAP kinase, Jun kinase, Elk and Jun biochemical activities, and 3) to determine the functional consequences of somatostatin action on these same components by determining whether somatostatin inhibits growth factor- or UV light-stimulated Elk-, Jun-, and Ets-mediated transcription.

Elucidation of the mechanisms involved in somatostatin action will not only further our understanding of the regulation of cell growth, and hence tumorigenesis, but may also lead to improved drug design for the treatment of breast cancer.

Body

In this section of the report, my research results are organized according to the Specific Aims set out by my original proposal. In addition to reporting on progress made in the past year, I have included some of the findings previously submitted in my 1998 annual report in order to better gauge the status of each Aim.

Aim 1: ***Develop a somatostatin-responsive breast cancer cell model system.***

Somatostatin receptors are present in very low numbers on most cell types, making analysis of signaling events difficult to measure by current techniques. As noted in the previous annual report, we initially proposed an approach that has been used by several investigators to elucidate signaling pathways from numerous types of receptors. The strategy was to make stable breast cancer cell lines that would overexpress sst2 and thereby amplify signaling events of this receptor in a physiologically-relevant cell type. We initially proposed to overexpress sst2 as it is believed to be the most prevalent subtype expressed in breast cancer tumors, and it is also the primary target of most of the commercially-available somatostatin analogs.

However, for reasons stated in the last report, we decided to pursue a different approach suggested by colleagues and reviewers, which was to identify a breast cancer cell line that shows a robust response to somatostatin via its endogenous somatostatin receptors. To accomplish this, several breast cancer cell lines were obtained from the University of Colorado Cell Culture Core Facility, which differed in estrogen receptor (ER) and EGF receptor status. They included the MCF-7, T47D, MDA 231 and ZR75.1 cell lines. The current literature contains few studies that have assessed somatostatin receptor expression in breast cancer cell lines, not all of the cell lines we have chosen have been examined, and furthermore, variations may exist in the "same" cell lines due to clonal differences at different sites. Therefore, we felt the best approach was to perform an analysis of sst expression in these cell lines ourselves. A reverse transcriptase-polymerase chain reaction (RT-PCR) approach was chosen because, as mentioned above, ssts are present in very low abundance and they cannot be readily detected by Northern blot or RNAse protection assays. Total RNA was prepared from the four cell lines, reverse transcribed to cDNA and then amplified by PCR with oligonucleotides specific for each of the five sst subtypes. The quantity of PCR product for each reaction was normalized to an internal control (GAPDH) in an identical reaction mix. The results indicated that each of the four cell lines examined displayed a relatively unique expression pattern of ssts, and some cell lines express certain receptors to a higher degree than others. The information gained from this RT-PCR study led us to focus on the MDA231 cell line as our model system. MDA 231 cells expressed a relatively high amount of sst2 and sst5, and importantly, show a strong response to the somatostatin analog BIM23014, with respect to the growth factor-regulated signaling pathways that we are interested in studying (Figs. 1 and 2).

We chose to utilize the somatostatin analog, BIM 23014 (Biomeasure Inc.) in our studies because it is a potent, long-acting agent that is used clinically for breast cancer

treatment. We also wanted to identify which somatostatin receptor subtype was mediating the effects in our experiments and BIM is reportedly specific to sst2. However, there are discrepancies in the literature with regard to specificity so we have recently performed studies to determine the specificity of the somatostatin analog BIM23014 for each of the somatostatin receptor subtypes. Briefly, subtypes 1-5 were each individually transected into CHO cells, which lack endogenous ssts. By measuring inhibition of cAMP, a well established effect of all somatostatin receptors, we determined that the BIM analog activates both sst2 and sst5 to inhibit cAMP, while native somatostatin activated all five receptors and inhibited cAMP, as expected (Fig. 3). Since sst2 and sst5 are abundantly expressed in the MDA 231 cells, we feel that these cells, in combination with the relatively selective BIM analog, provide a very good model system to study the mechanisms of somatostatin's antiproliferative effects in breast cancer.

AIM 2: ***Determine whether somatostatin inhibits growth factor- or UV light-stimulated Raf kinase, MAP kinase, Jun kinase, biochemical activities.***

The next goal was to test the cell lines for their ability to mediate somatostatin inhibition of growth-factor regulated signaling pathways (Aim 2). As reported in the previous annual report, we initially focused on the p44/42 MAP kinase (MAPK) pathway. MAPK plays a central role in cell proliferation, and we have shown that this pathway is inhibited by sst2 in pituitary tumor cells (6). Additionally, recent reports have implicated the importance of the MAPK pathway in breast tumorigenesis and it has been shown to be deregulated and MAPK is hyperexpressed in breast carcinomas (7,8).

Figure 1 shows the results of a Western blot analysis of MDA 231 whole cell extracts after various treatments using an antibody that is specific for the active, phosphorylated form of MAPK (phospho-MAPK). Lane 1 is a positive control phosphorylated p44 MAPK protein purchased from New England Biolabs. Lane 2 is a negative control p44 MAPK protein that is not phosphorylated. As expected, the phospho-specific antibody detected the phosphorylated MAPK but did not cross react with a non-phosphorylated MAPK. Lane 3 is extract from MDA 231 cells that were cultured in DMEM plus 10% fetal calf serum (FCS). It is interesting to note that these cells show a very high level of phospho-MAPK. I have observed that this cell line grows very rapidly in culture, and it was derived from an aggressive tumor, therefore, there may be a correlation between it's rapid growth characteristics and the degree of phosphorylation of MAPK (7,8). Since the level of phospho-MAPK is so high in cycling MDA 231cells in complete media, the remaining samples (lanes 4-12) represent MDA 231 cells that were grown in media devoid of FCS. This strategy is commonly employed to increase the signal to noise ratio, and thus make it easier to detect changes in phospho-MAPK levels in response to somatostatin. Lane 4 represents cells that are cultured in media without FCS. As expected, cells cultured under these conditions have very low levels of phospho-MAPK that are undetectable with the phospho-specific MAPK antibody. However, addition of 25 nM EGF for 10 minutes leads to an approximately 20-fold increase in MAPK phosphorylation (Fig. 1, lanes 5 and 6) when compared to quiescent cells (lane 4). Since MDA 231 cells showed a strong sst5 signal by RT-PCR (Table 1), I used the somatostatin 28 (SRIF 28), which targets this receptor subtype, in lanes 7-9. In lane 7, SRIF 28 alone was added, and this had

no further effects on basal phospho-MAPK levels. When SRIF 28 was added 5 minutes prior to EGF, it had no effect on MAPK phosphorylation when compared to EGF alone (lanes 5 and 6). MDA 231 cells also express sst2, and therefore I used the sst2-selective agonist, BIM23014 in lanes 10-12. Again, BIM alone failed to repress steady-state phospho-MAPK (lane 7). However, in contrast to SRIF 28, BIM23014 almost completely abolished the ability of EGF to stimulate MAPK phosphorylation. Therefore, it appears that only sst2 in these cells is coupled to the inhibition of MAPK phosphorylation (lanes 11,12).

Additionally, we performed experiments to determine if BIM23014 activates the Jun n-terminal kinase (JNK) pathway. Previously, our lab and others have shown that somatostatin can induce apoptosis in breast cancer cells, and that this may be a major mechanism for its anti-cancer effects (9). The JNK pathway is one of several potential routes in the apoptotic process, and in conjunction with an inhibition of MAPK provides an attractive model for somatostatin's inhibition of cell growth. In this experiment, T47D breast cancer cells were treated with and subjected to Western blot analysis and probed with an antibody specific for phospho-JNK. The results are shown in Fig. 2. A time course treatment shows a modest (approximately 2-fold) and transient increase in JNK phosphorylation, peaking at 5 minutes (Fig 2, lane 3), and then rapidly diminishing to control levels by 15 minutes. In the past year, we have repeated this study in the MDA 231 cell line. The results are essentially the same as those seen in the T47D cell line, in that JNK activity is modestly elevated in MDA 231 cells after treatment with BIM23014 (data not shown). However, we have been unable to consistently observe a greater than 2-fold increase in JNK phosphorylation. According to colleagues here at UCHSC who work on this pathway, a 10- fold or greater increase in JNK activity is typically seen with pro-apoptotic agents. Therefore it is difficult at this point to assess the significance of a two-fold increase in JNK activity. It is possible that the modest increase in JNK activity is relevant and this may become apparent when we assay the effects of somatostatin on Jun-mediated transcription (see Aim 3, below).

To date, we have not assayed the effects of somatostatin on Raf kinase activity in MDA 231 cells. However, we have obtained the necessary reagents, a phospho-specific Raf kinase antibody (NEB), and the dominant-negative Raf plasmid construct, pRSV C4B raf-1, to accomplish this. These studies will be undertaken in the near future.

AIM 3: ***Determine the effects of somatostatin on growth factor- or UV light-stimulated Elk-, Jun-, and Ets-mediated transcription.***

In order to accomplish this Aim, we will utilize a transient transfection approach using constructs that code for fusion proteins of Elk, Jun, and Ets transactivation domains fused to the yeast Gal4 DNA binding domain. This allows us to focus on the transactivation potential of these factors, independent of DNA binding and complex heterodimerization systems. The reporter construct, 5X UAS TK-luc, contains five copies of a Gal4 binding site upstream of a minimal TK promoter and luciferase reporter gene.

Thus far, we have performed experiments with Elk-1 and Ets-1. The Gal4-Elk and 5X UAS plasmids were gifts from Dr. James Hagman at the National Jewish Center, Denver, CO. The data for the Elk-1 experiments are shown in Fig 4. Briefly, MDA 231 cells were transfected by electorporation with the Gal4-Elk, 5X UAS-luc, and CMV-βgal plasmids (the latter is used to control for transfection efficiency). The cells were then quiesced in DMEM plus 1% fetal calf serum (FCS) for 18 hrs. Cells were then treated with vehicle or 10nM BIM23014 for 30 mins. We have determined through optimization experiments that this is the time point required for BIM to activate it's receptor(s) and transmit it's inhibitory signal (data not shown). After 30 mins. the cells were then stimulated with either serum, to a final concentration of 15% v/v, or EGF (25nM), and returned to the incubator for 6 hrs. The cells were lysed, extracts prepared and luciferase activity was measured and corrected for βgal activity (expressed as mean relative light units). As shown in Fig 4, the 5X UAS construct has very little activity in low serum conditions. However, as expected, serum and EGF lead to dramatic increased in Elk activity to approximately 18-and 17-fold of control, respectively. In cells pretreated with BIM23014, the serum challenge led to an increase that was approximately 59% of serum alone levels (~40% decrease in activity compared to serum alone). EGF alone stimulated Elk to levels fairly similar to serum, but in this case, pretreatment with BIM caused a much greater decrease in Elk activity, approximately 20% of EGF alone (80% decrease). Thus, BIM23014 is capable of diminishing Elk transcriptional activity stimulated by serum or EGF, but to a much greater extent with EGF. The reason for this is perhaps that serum contains a complex mixture of growth factors and other stimulators of Elk, only a few of which may be inhibited by somatostatin.

The Gal4-Ets plasmid construct was a generous gift from Dr. Bohdan Wasylyk, and consists of the Ets-1 transactivation domain I and regulatory domain II fused to the yeast Gal4 DBD. The results of the Ets-1 experiments are given in Fig 5. These experiments were performed essentially the same as those for Elk, described above, except that we used fibroblast growth factor-2 (FGF-2) as the growth factor stimulus. Our laboratory has shown that FGFs are important components of signaling pathways that culminate in the activation of Ets factors (11). FGFs are also critical growth factors in breast cancer that are often found to be overexpressed (5). Again, the level of activity of the Gal4-Ets/5XUAS combination is very low in 1% serum conditions. And, as expected, 15 % serum and FGF-2 (2 ng/ml) caused increased Ets activity to approximately 20- and 16-fold versus control, respectively (Fig 5), However, BIM pretreatment caused a much smaller dimunition of these stimuli than for Elk-1. It is possible that the effects of BIM on FGF-stimulated Ets-1 activity is statistically significant, but we have yet to perform a statistical analysis on these data.

We have yet to determine the effects of somatostatin on Jun-mediated transcription. In initial experiments, the Gal4-Jun construct we obtained has not been responsive to either UV light or osmotic stress, which are known activators of Jun. We are currently sequencing the plasmid insert to verify that it is correct, and we will request a new plasmid if necessary.

Other studies stemming from this proposal

As a postdoctoral fellow, one of the main goals of my training is to eventually obtain a position as an independent investigator. Towards this end, my mentor, Dr. Gutierrez-Hartmann, has encouraged me to explore novel projects related to somatostatin and breast cancer that may provide the basis for future funding opportunities, and also to have a project that is my own to take with me. Below, I briefly describe some very recent research ideas that I am exploring. I believe these studies are complementary to the goals of this proposal and will not interfere with the timely completion of the remaining aims in my Statement of Work.

The results described above indicate that somatostatin can inhibit the transcriptional activity of at least two members of the Ets family of transcription factors, Ets-1 and Elk-1. Ets factors have important roles in proliferation and differentiation of cells and in oncogenic transformation (10). Recently, a novel epitheial-specific human Ets factor, ESX (or ESE-1) has been identified. ESX mRNA is upregulated in certain breast cancers, and a positive feedback loop may exist between the HER2/neu proto-oncogene and ESX in human breast cancer cell lines (12). As a logical extension of the current studies, I would like to determine if somatostatin controls breast cancer cell growth and perhaps transformation, by diminishing ESX expression, which is aberrantly overexpressed in these cells. Specifically, I would like to determine if somatostatin affects ESX expression at the level of transcription and/or protein levels.

In order to achieve this, we would require the ESX promoter, cloned into a luciferase reporter construct, the ESX cDNA for use as a probe of Northern blots, and a good antibody to ESX to monitor protein levels in response to somatostatin treatment.

We are currently attempting to clone the ESX promoter.

We have isolated the ESX cDNA from T47D cells by RT-PCR, and have cloned thegene into a mammalian expression vector with an HA-epitope tag. This cDNA can be used in Northern blot experiments, but as shown in Fig. 6, the mRNA for ESX is expressed at very low levels, requiring polyA+ RNA isolation techniques. This, however, should not be a detriment to studies seeking to assess the effects of somatostatin treatment on steady-state ESX mRNA levels. We hope to begin preliminary studies soon.

As there are no ESX antibodies currently available, commercially or otherwise, our laboratory is working with Affinity Bioreagents of Golden, Colorado to develop a polyclonal antibody to human ESX. We have tested the initial crude antibody preparations against GST-ESX fusion proteins produced in bacteria and the results are shown in Fig. . extracts and The antibody still is rather crude, with too much cross-reactivity, but we are confident that with further purification, we will have a good tool with which we can pursue these studies.

Key Research Accomplishments

* Performed comprehensive assessment of the expression levels of all five somatostatin receptor subtypes in several breast cancer cell lines.

*Demonstrated that the MAPK signaling pathway, which is overexpressed in certain aggressive breast cancers, is strongly inhibited by the somatostatin analog BIM23014, most likely via the type 2 somatostatin receptor (sst2).

* Identified N-terminal Jun kinase (JNK) pathway as a potential mechanism by which somatostatin exerts its pro-apoptotic effects in cancer cells.

*Demonstrated that transcription potency of factors downstream of the Ras/Raf/MAPK mitogenic pathway are inhibited by BIM23014. Specifically, EGF and FGF-stimulated Ets-1 and Elk-1 activity.

Reportable Outcomes

Abstracts and manuscripts

*Abstract: John J. Tentler, Kristin Eckel and Arthur Gutierrez-Hartmann. "The somatostatin analog, BIM23014 inhibits the growth factor/MAPK/Elk-1 Pathway in MDA231 breast cancer cells". University of Colorado Annual Research Forum, Department of Medicine.

*Abstract: John J. Tentler and Arthur Gutierrez-Hartmann. "Somatostatin Inhibits Breast Cancer Cell Growth via Multiple Signaling Pathways". DoD Era of Hope Annual Meeting, Atlanta, Ga. June 8-12, 2000.

*Manuscript: Tentler, JJ, Diamond, SE, Gutierrez-Hartmann, A. Control of Pituitary Gene Expression. In: *Pituitary Diseases:Diagnosis and Treatment.* JD Baxter, S Melmed and N New, Eds. Humana Press, Totowa NY In press, 2000.

* Manuscript in preparation: John J. Tentler, Kristin Eckel and Arthur Gutierrez-Hartmann. "Somatostatin Inhibits Breast Cancer Cell Growth via Multiple Signaling Pathways".

Funding and employment opportunities

* Funding support for 1 year on UCHSC Endocrine Division NIH Training Grant

*Promotion in UCHSC Division of Endocrinology from Postdoctoral fellow to Instructor

*Currently applying for faculty research positions at several institutions and biotech companies.

Conclusions

In this report, I describe the progress made thus far on the elucidation of the signaling components mediating the anti-proliferative effects of somatostatin and its analogs in breast cancer cells. After testing several breast cancer cell lines for somatostatin receptor expression and response to somatostatin analogs, we have chosen the MDA 231 cell line for our studies. This cell line expresses relatively high levels of the sst2 and sst5 receptor subtypes, which we have empirically determined to be the primary targets for the long-acting somatostatin analog, BIM23014 used in our experiments. Using this system, we have shown that the growth factor-stimulated MAPK pathway is strongly inhibited by the somatostatin analog, BIM23014. We have also shown that in these cells, activation of sst2, but not sst5, leads to inhibitory signals that block the EGF stimulation of the MAPK pathway. Furthermore, we have shown that the transactivation potential of two members of the Ets family of transcription factors, Elk-1 and Ets-1 are inhibited by BIM23014, The effects on Elk-1 in response to EGF are the most dramatic. Since EGF and the MAPK pathway have been shown to be critical in breast cancer tumorigenesis, these findings offer significant insights into the antiproliferative actions of somatostatin. Additionally, some preliminary data in T47D and MDA231cells indicates a possible role of the JNK pathway in the control of cell proliferation as well, though the significance of this activation to somatostatin's antiproliferative effects has yet to be assessed.

Future studies include mapping the precise point(s) in the MAPK pathway where sst2 and sst5 signals act (Aim 2), and investigating the potential role of phosphatases, including MAPK phosphatase and PTP1B in somatostatin's inhibition of MAPK. I will be completing studies on raf kinase activity (Aim 2) and Jun transactivation activity (Aim 3) when technical details of these experiments and problem areas are worked out. Also, some of the experiments presented in this report will need to be repeated before publishing the results. I will also be exploring the possible effects of somatostatin on the expression of a novel Ets factor implicated in breast cancer progression, ESX. Finally, I also plan to assess the effects of somatostatin on various cell cycle regulating factors, which may be critical effectors of the somatostatin inhibitory signal.

References

1. Lamberts SWJ, Krennig EP, Reubi J-C. The role of somatostatin and its analogs in the diagnosis and treatment of tumors. Endocrine Reviews 1991; 12: 450-482.

2. Reubi J-C and Laissue JA. Multiple actions of somatostatin in neoplastic disease. Trends Pharm Sci 1995; 16:110-115.

3. Bell GI, Reisine T. Molecular biology of somatostatin receptors. Trends in Neurosciences 1993; 16:120-131.

4. Xu Y, Song J, Berelowitz M, Bruno JF. Estrogen regulates somatostatin receptor subtype 2 messenger ribonucleic acid expression in human breast cancer cells. Endocrinology 1996; 137:5634-5640.

5. McGuire WL, Dickson RB, Osborne CK, Salomon D. The role of growth factors in breast cancer. Breast Cancer Research and Treatment 1988, 12:159-166.

6. Tentler J, Gutierrez-Hartmann A. Signaling via the type 2A somatostatin receptor (sst2A) inhibits growth factor stimulated MAP kinase activity in GH4 pituitary cells. J Endocrinol Invest. 1997; 20:77.

7. Coutts AS, Murphy LC. Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 1998; 58(18):4071-4.

8. Sivaraman VS, Wang H, Nuovo GJ, Malbon CC. Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest 1997; 99(7):1478-83.

9. Candi E, Melino G, DeLaurenzi V, Piacentini M, Guerrieri P, Spinedi A, Knight R. Tamoxifen and somatostatin affect tumours by inducing apoptosis. Cancer Letters 1995; 96:141-145.

10. Wasylyk, B., Nordheim, A. Ets transcription factors: partners in the integration of signal responses. In: Papavassiliou ed. Transcription Factors in Eukaryotes. Landes Bioscience, 1997 p251-284.

11. Schweppe, R., Frazer-Abel, Gutierrez-Hartmann, A., Bradford, A. Functional components of fibroblast growth factor (FGF) signal transduction in pituitary cells. Jour Biol Chem 1997 272:30852-30859.

12. Chang, C., Scott, G., Kuo, W., Xiong, X., Suzdaltseva, Y., Park, J., Sayre, P., Erny, K., Cllins, C., Gray, J., Benz, C. ESX: A structurally unique Ets factor overexpressed early during human breast tumorigenesis. Oncogene 1997 14:1617-1622.

Figure 1.

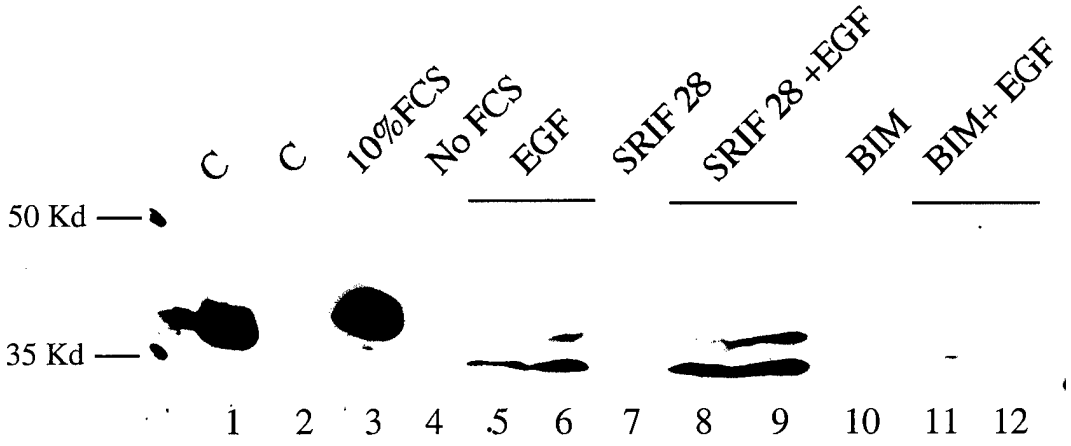
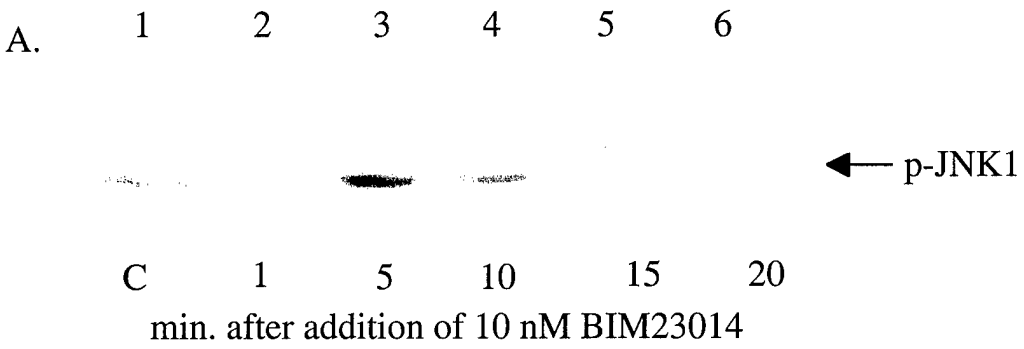


Figure 1: Western blot analysis of MDA231 breast cancer cell extracts treated as shown above and as described in Body text. Equal protein amounts (50 μg) were separated on 12% SDS-PAGE, blotted onto Immobilon and probed with an antibody specific for the phosphorylated form of MAPK.

Figure 2.



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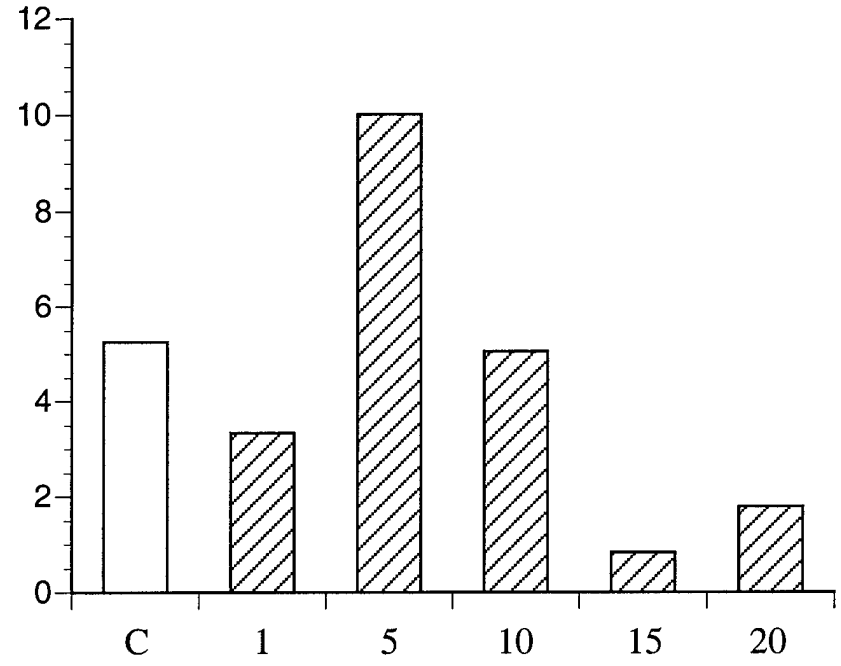

Figure 2. The somatostatin analog BIM23014 induces a transient increase in JNK phosphorylation. **A**. Western blot analysis of T47D breast cancer cells treated with vehicle (C) or 10nM BIM23014 for the indicated time points, and as described in the Body text. Extracts were prepared, and equal protein amounts (50 μg) were separated on a 12%SDS-PAGE, transferred to Immobilon and probed with anatibody specific for phosphorylated JNK. **B**. Densitometric analysis of the blot shown in **A**. Values are corrected for loading differences.

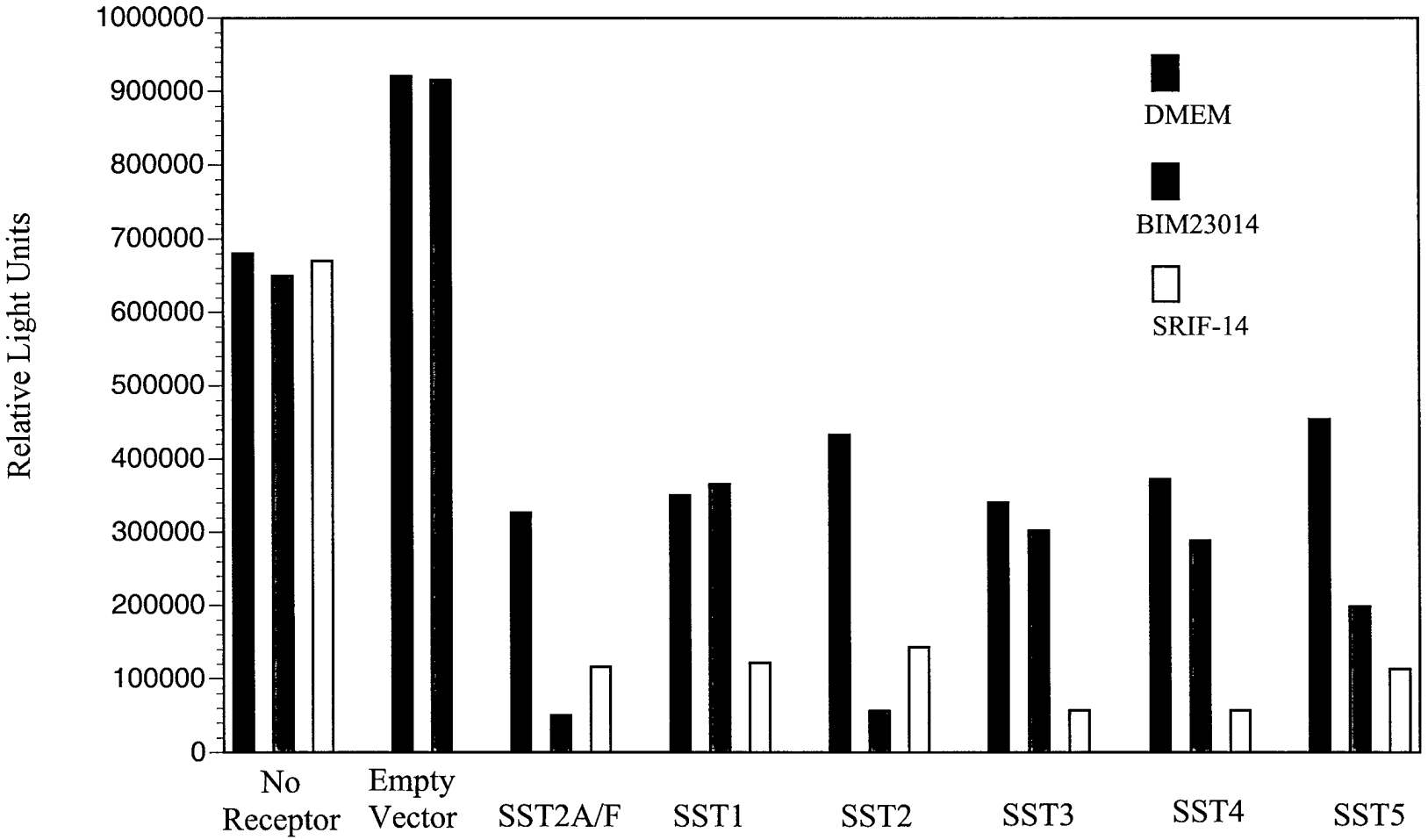

Figure 3: Somatostatin receptor subtype inhibition of the cyclic AMP/CREB-responsive α-glycoprotein subunit promoter. CHO cells, lacking endogenous sst's were co-transfected with each of the sst subtypes (sst 1-5), and the α-glycoprotein subunit promoter fused to the luciferase reporter gene. Cells were pretreated with either vehicle (DMEM) or BIM23014 or the native ligand, somatostatin 14 (SRIF-14). Light units corrected for total protein are shown. sst2A/F is a FLAG epitope-tagged version of sst2.

Figure 4

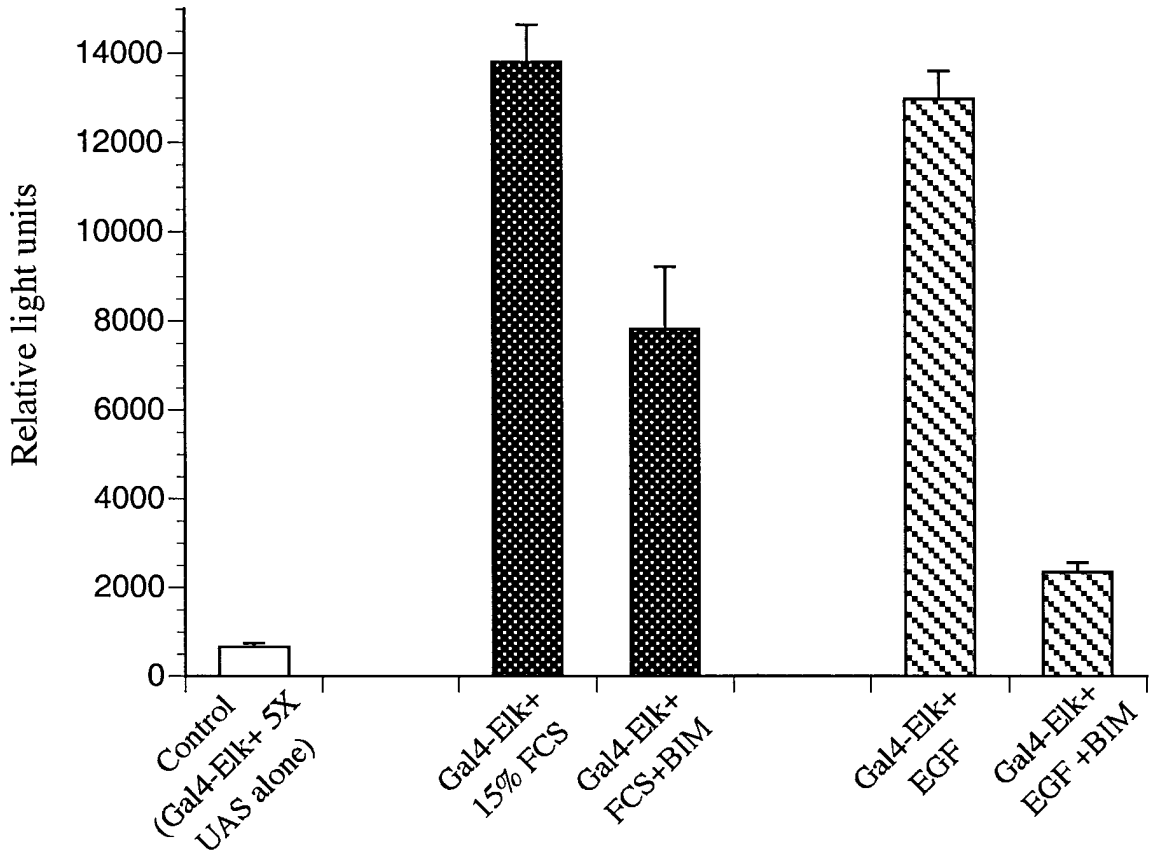

Figure 4: Effects of BIM 23014 on serum and EGF-stimulated Elk-1 transcriptional activity. MDA 231 cells were transiently transfected by electroporation with a Gal4-Elk-1 fusion and a 5X UAS construct, and then incubated for 18 hrs. in DMEM with 1% FCS. Some cells were pretreated with 10nM BIM23014 for 30 mins., then stimulated with FCS (15% v/v final concentration) or EGF(25nM) and incubated for 6 hrs. Values represent mean relative light units +/- SD. n=6 per experimental group.

Figure 5

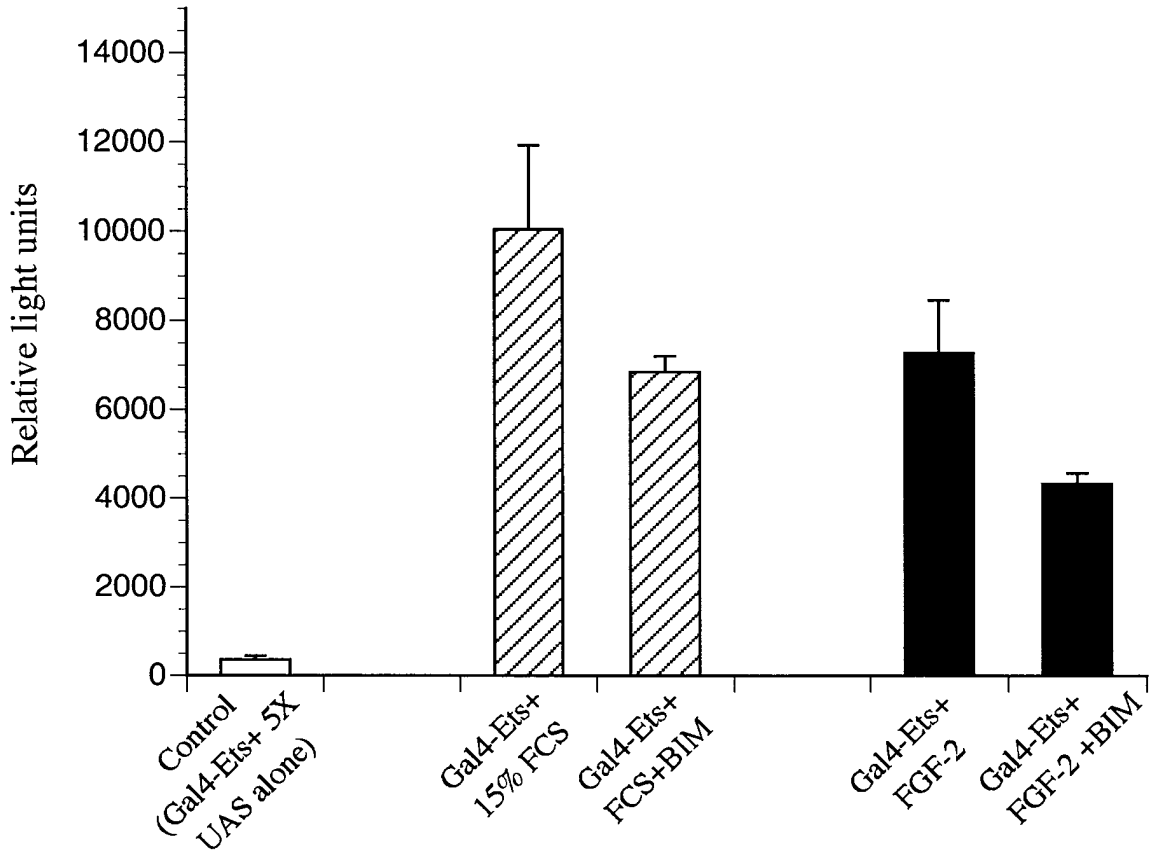

Figure 5: Effects of BIM 23014 on serum and FGF-2-stimulated Ets-1 transcriptional activity. MDA 231 cells were transiently transfected by electroporation with a Gal4-Ets-1 fusion and a 5X UAS construct, and then incubated for 18 hrs. in DMEM with 1% FCS. Some cells were pretreated with 10nM BIM23014 for 30 mins., then stimulated with FCS (15% v/v final concentration) or FGF-2 (2 ng/ml) and incubated for 6 hrs. Values represent mean relative light units +/- SD. n=3 per experimental group.

Figure 6

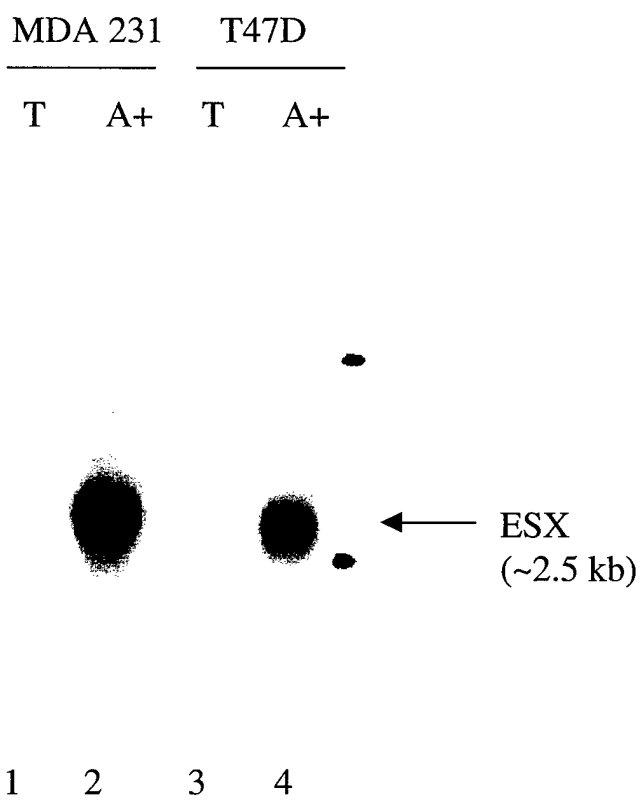

Figure 6: Northern blot analysis of ESX mRNA in two breast cancer cell lines. Total (T) and poly A+ RNA (A+) was isolated from MDA 231 (lanes 1+2) and T47D (lanes 3+4) cell lines, and probed with a ^{32}P-labeled full length ESX cDNA. Twenty μg of total and 5 μg poly A+ were run for each cell line. Exposure time is approximately 18 hours.

Figure 7

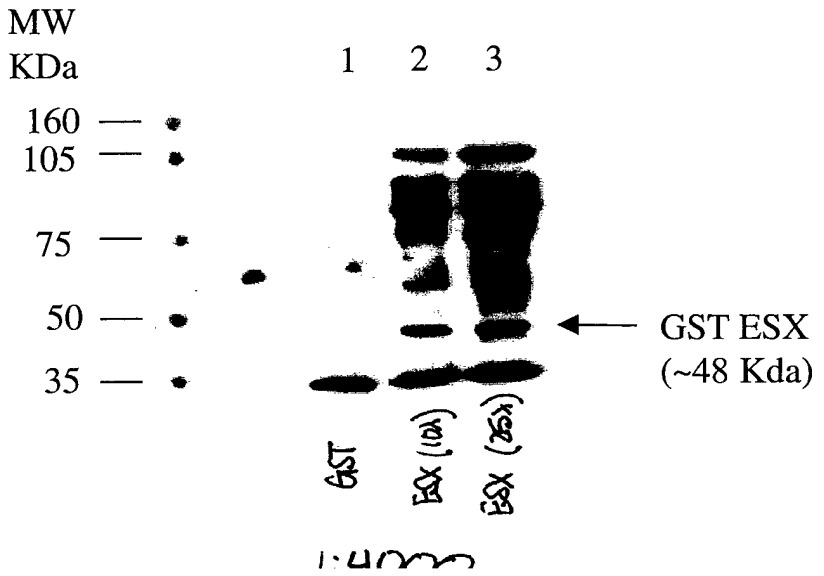

Figure 7: Western blot analysis of GST-ESX. A crude anti-ESX polyclonal antibody was tested on bacterial extracts of GST alone (25 µl, lane 1) or 10 µl (lane 2) and 25 µl (lane 3) of GST-ESX extracts. Proteins were separated on a 12% denaturing polyacrylamide gel and transferred to nitrocellulose.